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# A Preliminary Antidepressant Assessment of a Novel Piperoyl Amide (Y3) Isolated and Elucidated from *Piper nigrum* Drupes Extract, Justifying Interaction Over MAO-A enzyme: An *in vivo*, *in vitro* and Molecular Docking Approach

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## ABSTRACT

This research aims to confirm the preliminary antidepressant activity of a newly discovered piperoyl amide (Y3) obtained from the extract of Piper nigrum (P. nigrum) drupes, through the inhibition of MAO-A enzyme. Column chromatography was employed for isolation and purification of Novel piperoyl-alkyl amide (Y3) which was eluted in a solvent mixture of Chloroform-Methanol (97:3). Structure elucidation was performed by implying spectroscopic techniques. Invitro in-comparison MAO activity assessment was performed between Y3 and piperine further confirmed by Molecular docking via Auto Dock Tools 1.5.6, Vina, and PyMOL. Invivo antidepressant activity was evaluated by the Despair Test in albino mice. Novel piperoyl-alkyl amide (Y3) was isolated and purified by column chromatography and elucidated spectroscopically. The antidepressant activity of Y3 at 10 and 20 mg/kg doses shown a significant decrease in immobility-time (IT) in contrast to stressed-control group at 107.0±7.6 sec ($P<0.05$) and 96.0±7.6 sec ($P<0.01$), respectively. Y3 shown a decent MAO-A inhibitory activity but lower than piperine showing $IC_{50}$=20.24 µM, $V_{max}$=1.375±0.3 nM/min/mg, $K_m$=1.379×$10^{-16}$ µM with a stronger interaction and better affinity (-12.6 kcal/mol) towards the active-site of MAO-A. Y3 was isolated and elucidated as (12E,14E)-15-(benzo[d] [1,3] dioxol-5-yl)-N-ethylpentadeca-12,14-dienamide. It proved a good antidepressant at 10 and 20 mg/kg doses. Molecular docking over MAO-A and QSAR studies depicted a better affinity score of Y3 than Piperine.

***Keywords:*** Piperoyl amide, Structure elucidation, antidepressant activity, molecular modeling, Piperine

## INTRODUCTION

*Piper nigrum*, known as black pepper, is native to southern India and widely used as a spice worldwide. In recent decades abundant research had revealed the promising biological characteristic of Piperine alkaloid, a chief phytoconstituent, imparting assorted pharmacological properties especially Neurological activities. Piper nigrum extract was reported for prominent antidepressant activity. Activity imparted is not from a specific isolated phytoconstituent only but is a concerted outcome of phytoconstituents concoction, which indicates towards more than one compounds of antidepressant attribute[1], hence our aim was dedicated to extract, purify, characterize and rationalize any

novel compound, with receptor interaction at molecular modeling level. Piperine was screened previously as an antidepressant by inhibiting MAO-A, and B [2] and enhancing the 5HT level [3]. Although several studies have been done so far on MAO inhibition but still a clear concept of MAO inhibition is needed to be updated. Monoamine oxidases (MAO) enzymes catalyzes catecholamines metabolism via oxidative deamination, deficiency of these catecholamines causing depression and parkinsonism [4]. MAO-A functions at the periphery metabolizing adrenaline, dopamine, noradrenaline, and serotonin taking part in the regulation of emotions and behavioral activities and have depicted a major role in depression. MAO-B functions in the brain, which is mainly metabolizing dopamine, has shown a major role in Parkinson [2], hence MAO-A was selected for Molecular modeling in current research. Till date several alkaloids and phytoconstituents reported from *P. Nigrum*, some major compounds are Pellitorine, piperidine and isobutyl amide derivatives, paprazine, N-feruloyltyramine, piperolactam D, piperlonguminine, piperine, piperettine, sylvamide cepharadione, guineensine, pipercyclobutanamides, and pipericide [5-7]. The objective of this study is to isolate, elucidate, and assess the potential antidepressant effects of a newly discovered piperoyl amide (Y3) from the Piper nigrum drupe. Additionally, the study investigates the molecular docking interaction between Y3, piperine, and MAO-B.

## MATERIALS & METHODS

### *Material, instrumentations, Chemicals, and Software's*

A gift sample of the standardized hydroalcoholic extract of Piper nigrum drupe was acquired from Volhart Healthcare Pvt Ltd in Lucknow, India, and its authentication was carried out through spectrophotometry. Structure elucidation was accomplished by implying spectroscopic techniques, NMR (500 MHz): DRY 400, (Bruker, Massachusetts, United States), FT/IR-5000 (JASCO Inc., Easton, USA); ESI-MS ionization, 70 eV (JEOL-JMS-DX 303, Massachusetts, United States). Silica gel-G, 60-120 mesh, Piperine, acetone, chloroform, ethanol, methanol, and petroleum (Sigma Aldrich, Bayoni-Saudi Arabia). For molecular docking MAO-A enzyme (PDB ID-2BXR) [8] was utilized, further AutoDock Tools 1.5.6, Vina [9] and PyMOL were utilized for Protein and Ligand PDBQT generation and Molecular visualization [10]. Statistical evaluations were performed via One-way nonparametric ANOVA, posttest Dunnett's Multiple Comparison was employed for invivo DT and nonlinear regression, Michaelis-Menten for enzyme kinetics by GraphPad (V5.02, SanDiego, CA, USA) $P<0.05$ contemplated statistically significant.

### *Isolation of Y3*

100 grams of the hydroalcoholic-extract was silica adsorbed (60-120 mesh), dried, and the column-packed in pet-ether (60-80 °C) and employing Chloroform-Methanol (97:3) as elution mixture.

### *Invivo* antidepressant activity

The Despair Test (DT) model was selected for screening antidepressant activity. Albino mice of weight ranging from 20–25 g were selected after approval from the institutional ethical committee. The animals were kept under room temperature (25±2°C) and, 12-hour light/ dark cycles with appropriate food and water. 6 groups, each of 6 animals were divided for the study. Group 1 was set to be devoid of any stress swim training with no drug and was labeled as control, whereas other groups were kept for stress training daily for 15 minutes for 7 days. Group 2 was not given any drug and labeled as stress control. Each drug was given through the i.p route in saline (0.9%) dispersed in tween 80 (0.1%). Group 3 and 4 received Y3 5 and 6 received Piperine at 10 & 20mg/kg, two times a day, for 7 days. After $7^{th}$ day, the main DT was conducted but a pretest for 15 minutes was also conducted 6 hrs ago,

where immobility time (seconds) in 300 seconds was estimated [11].

### *MAO Activity of Y3 Vs Piperine*

A modified method from were adopted for estimating MAO-A activity of Y3 and Piperine. Mitochondrial portion extracted from mice were fluorometrically assessed by the substrate kynuramine. $V_{max}$ and $K_m$ were calculated by nonlinear regression using Michaelis-Menten enzyme kinetics.

## RESULT AND DISCUSSION

Y3 structure was elucidated by IR, NMR, and Mass spectroscopy (Table 1). IR exhibited significant absorption for; amide group (amide N-H 3497 $cm^{-1}$, C=O 1632 $cm^{-1}$), trans-ene (995 $cm^{-1}$), aromatic stretching (3229 $cm^{-1}$ C-H, 1610, 1654 $cm^{-1}$ C=C), aliphatic $CH_2$, (2857, 2927 $cm^{-1}$), =C-O-C, stretching (1132, 1251 $cm^{-1}$), Benzene CH (1036 $cm^{-1}$), Trans-CH (995 $cm^{-1}$), C-O, stretching (931 $cm^{-1}$). Mass spectra confirmed its molecular weight *m/z* 386 $[M+H]^+$, and molecular formula as; $C_{24}H_{35}NO_3$ and identified it as a piperine kind amide, besides the main molecular peak, two other clear peaks were obtained at *m/z* 356 $[M\text{-}NHCH_2CH_3]^+$ and 341 $[M\text{-}CH_2CH_3]^+$ implying as ethylamido-piperoyl derivative. NMR ($^1H$) spectrum revealed deshielding of three aromatic ring protons, i.e *ortho*-coupled doublet (2') at δ 7.21 (*J*=2.5 Hz), other *ortho*-coupled double-doublet (6') at δ 7.14 (*J*=7.5, 2.5 Hz) and at δ 6.94 (*J*=7.5 Hz) a doublet of *meta*-coupled (5'). Two outer proton double doublets (12, 15) were shown at δ 5.76 (*J*=12.5, 6.5 Hz) and 7.01 (*J*=12.5,6.5 Hz), another two inner proton doublets (13, 14) at δ 6.29 and δ 6.71 with a high coupling constant of 12.5 Hz, as high coupling constant engrave for trans-ene protons confirming 12, 13, 14 and 15 four trans Hydrogens. Dioxomethylene protons also show a desheilded signal at δ 6.03. One-proton multiplet at δ 2.18 and triplet at δ 3.12 was characterized for Methylene (1''), Methyl group (2''), and terminal ethyl chain linked to amido group. A long broad peak at 1.30 characterized similar protons of seven methylene (4,5,6,7,8,9,10). NMR ($^{13}C$) characterized amide Carbon ($CONH_2$) at δ 171.6 (C-1); benzene carbons ranged from δ 148.5-106.7 and dioxomethylene (O-$CH_2$-O) carbon at δ 101.3. The two outer trans-ene carbons (12, 15) are shown to peak at 135.9 and 136.3 whereas, another two inner carbons (13, 14) were shown at 128.4 and 130.3. Lastly methyl carbon (C-2'') is shown to peak at 14.9. This all confirmed Y3 as novel piperoyl amide with structure name (12E,14E)-15-(benzo[d][1,3] dioxol-5-yl)-N-ethylpentadeca-12,14-dienamide. (Figure 1)

Monoamine oxidases (MAO) are a special category of oxidoreductase flavin-containing enzymes liable for the destructive catalysis of catecholamines by a mechanism known as oxidative deamination, where the amine group is replaced by a carbonyl group and ammonia [equation 1]. Catecholamines are functional neurotransmitters including adrenaline, dopamine, noradrenaline, and serotonin. MAO enzyme is classified into two; MAO-A and B. MAO-A functions at the periphery metabolizing adrenaline, dopamine noradrenaline and serotonin, participating in the regulation of emotions and behavioral activities and have shown a major role in depression. Whereas MAO-B functions inside the brain, metabolizing Dopamine mainly and presents a major role in Parkinson and Depression. Overall, it is certain that the deficiency of these catecholamines causes neurological diseases.

$R\text{-}C_6H_5CH_2NH_2+O_2+FAD+H_2O=R\text{-}C_6H_5CHO+HOOH+FADH_2+NH_3$ [1]

Piperine and similar analogs have already shown a good MAO-A inhibitory activity. MAO inhibition results in reduced metabolism of catecholamines, and improved behavioral activities, that accomplished by antidepressants and Anti-parkinsonism drugs [4]. The behavioral nature of mice can successfully be studied by the DT as it evaluates mice behavioral

despair characteristics. In DT, when mice or rats are compelled to swim in an unescapable restricted area, induces immobility, signifying behavioral peculiarity and imitates a situation of despair [11]. Despair condition is the key factor to evaluate Alzheimer's, and depression [12, 13], hence this model proved as best, for estimating the effectiveness of a drug towards the behavioral functions and may further implied for evaluation of other neurological activities.

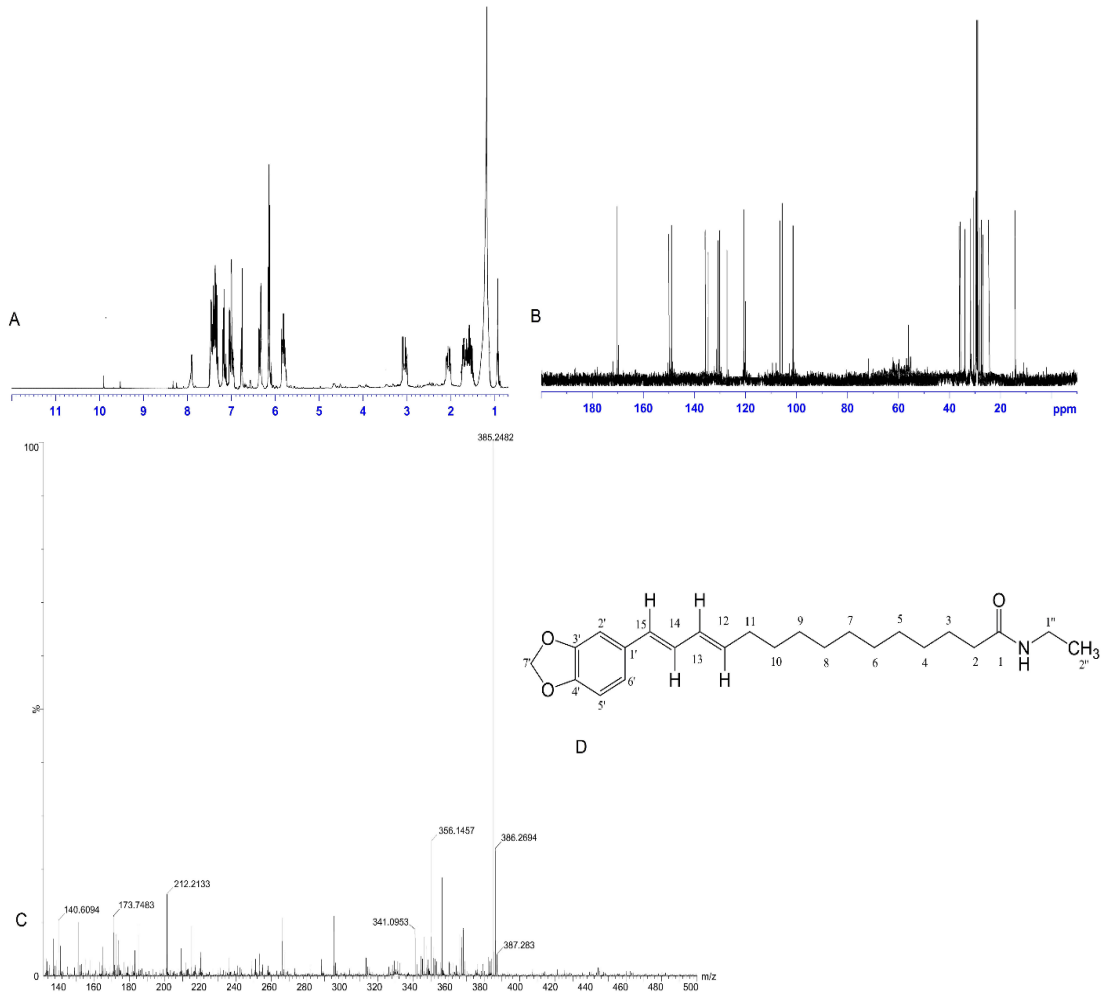


**Figure 1. A. 1H-NMR Spectrum B C-NMR spectrum, C. MASS spectrum, D. Structure elucidated of novel piperoyl amide (12E,14E)-15-(benzo[d][1,3]dioxol-5-yl)-N-ethylpentadeca-12,14-dienamide isolated from *Piper nigrum* drupes.**

**Table 1: $^{1}$H and $^{13}$C NMR data values of Y3 with carbon positions**

| Position | Moiety | $^{1}$H-NMR | $^{13}$C-NMR |
|---|---|---|---|
| 1 | C=O | - | 171.6 |
| 2 | $CH_2$ | 2.18 m | 36.5 |
| 3 | $CH_2$ | 1.56 m | 25.7 |
| 4 | $CH_2$ | 1.30 brs | 28.5 |
| 5 | $CH_2$ | 1.30 brs | 28.7 |
| 6 | $CH_2$ | 1.30 brs | 29.6 |
| 7 | $CH_2$ | 1.30 brs | 29.6 |
| 8 | $CH_2$ | 1.30 brs | 29.7 |
| 9 | $CH_2$ | 1.30 brs | 29.7 |
| 10 | $CH_2$ | 1.30 brs | 30.1 |

| | | | |
|---|---|---|---|
| 11 | $CH_2$ | 2.21 m | 33.9 |
| 12 | Trans-CH | 5.76 dd (12.5,6.5) | 135.9 |
| 13 | Trans-CH | 6.29 d, (12.5 ) | 128.4 |
| 14 | Trans-CH | 6.71 d (12.5) | 130.9 |
| 15 | Trans-CH | 7.01 dd (12.5, 6.5) | 136.3 |
| 1’ | Benzene-C | - | 130.3 |
| 2’ | Benzene-CH | 7.21 d (2.5) | 106.7 |
| 3’ | Benzene-C | - | 148.5 |
| 4’ | Benzene-C | - | 148.2 |
| 5’ | Benzene-CH | 6.94 dd (7.5) | 108.3 |
| 6’ | Benzene-CH | 7.14 dd (7.5, 1.5) | 122.6 |
| 1’’ | $CH_2$ | 3.12 m | 36.2 |
| 2’’ | $CH_3$ | 1.04 t | 14.9 |
| | NH | 7.9 | |
| | O-$CH_2$-O | 6.03 brs | 101.3 |

In DT Control group forced swimming was not applied for six days, Immobility time (IT) of 107.2±6.8 secs were estimated on 7th day was, while stressed control showed a significantly ($P<0.05$) increased immobility time to 132.7±6.9 secs in disparity to control group. Group 3 (Y3-10 mg/kg) and group 4 (Y3-20 mg/kg) shown a reduced IT significantly at 107.0±7.6 sec ($P<0.05$) and 96.0±7.6 sec ($P<0.01$), whereas Piperine reduced the IT in group 5 (PIP-10 mg/kg) nonsignificant to 114.0±4.7 sec and significantly to 99.83±6.9 secs ($P<0.05$) in 6$^{th}$ group (Y3-20 mg/kg) in disparity to the stressed control group (Figure-2). Y3 shown a better response at 10 and 20 mg/kg in contrast to Piperine, and well explained by QSAR and molecular docking approach. (Figure 2 A)

Y3 predicted an appreciable good MAO-A inhibition ($IC_{50}$=20.24µM) but lower than piperine ($IC_{50}$=11.32µM). To elaborate the inhibition activity a nonlinear regression analysis using Michaelis-Menten enzyme kinetics was employed which concluded Y3 and Piperine founding MAO inhibition at different concentration, $K_m$ of $1.379\times10^{-16}$ and $1.433\times10^{-16}$ µM whereas the reaction rate ($V_{max}$) was estimated as 1.375±0.3 and 1.120±0.2 nM/min/mg. (Figure 2 B) $V_{max}$ (maximum rate) and Km (substrate concentration) are two major constants for depicting enzyme kinetics and enzyme-drug interaction. $K_m$ is described as the minimum substrate concentration needed to reach half of maximum rate ($V_{max}$), Y3 and Piperine both have shown MAO inhibition at different substrate concentration, Km of 1.379×10-16 and 1.433×10-16 µM at $V_{max}$ of 1.375±0.3 and 1.120±0.2 nM/min/mg. Studies show of increased $K_m$ is an indication of competitive inhibition, suggesting piperine, a better competitive inhibitor than Y3. [14] $V_{max}$ predicts of allosteric interaction towards substrate, as a low value is shown by piperine predicting a better allosteric inhibition in contrast to Y3. [15] DT shown a finer antidepressant assessment of Y3 at 10 and 20 mg/kg doses and polyene nature of Y3 maybe the rationale. Our previous studies confirmed similar compounds had alleviated CNS ailments via reducing oxidative stress. [12, 13, 16]

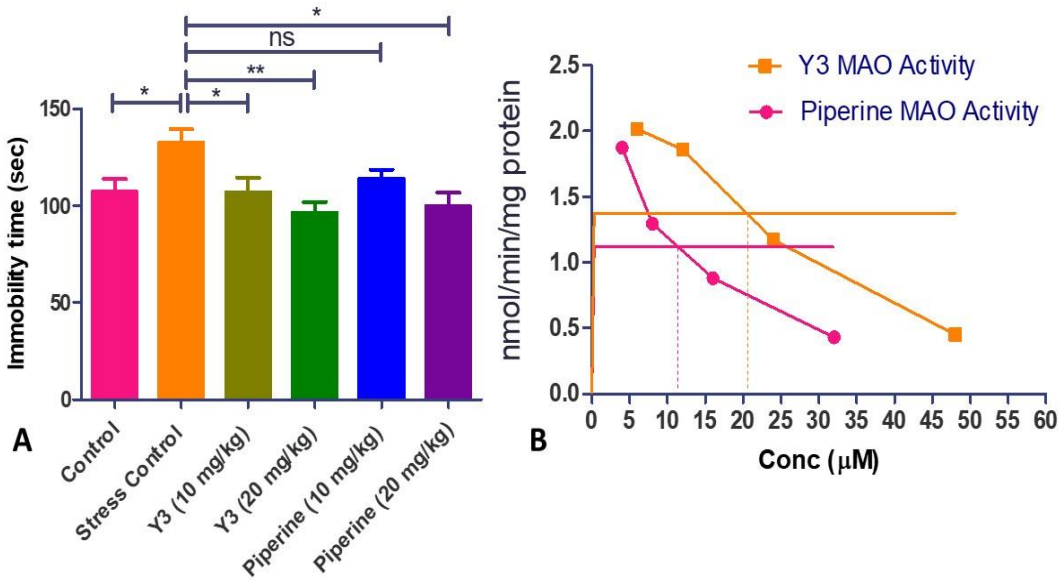


**Figure 2: A. Despair Test performed on the 7th, on *albino* mice (n=6). Statistical studies were done using one-way nonparametric ANOVA, posttest Dunnett's Multiple Comparison, Immobility time (secs) was stated in mean ± S.E.M. Significance represented as *P<0.05, and **P<0.001. B. nonlinear-regression analysis using Michaelis-Menten enzyme kinetics, implicating $IC_{50}$ of Y3=20.24 µM and Piperine 11.32 µM, respectively.**

A comparison between Piperine and Y3 was made on the receptor site to depict molecule and receptor interaction. Both Piperine and Y3 act on the same receptor pocket but different active sites. (Figure 3) Y3's affinity was calculated to -12.6 kcal/mol whereas Piperine showed an affinity of -7.5 kcal/mol, suggesting Y3's stronger interaction than piperine over the binding site. One oxygen of dioxole ring in Piperine interacted with Lysine-190 and active water-2141 at an intermolecular distance of 3.2 Å, whereas the carbonyl group interacted by four binding sites, two by active waters-2143, 2146 (2.9 Å), and other two with Threonine-196 (3.4 Å). On the other hand, oxygens of dioxole ring in Y3 interacted with the active water-2081 measuring distance of 2.3 and 2.4 Å, whereas the carbonyl group interacted both with active water-2086 at (3.4 Å) and Asparagine-116 (2.1 Å). (Figure 4)

At the molecular level, Y3 imparted low Gibbs energy at 241.93 kJ/mol in comparable to piperine 268.19 kJ/mol which suggests the perfect fit of ligand more effectively on the receptor site. cLog P gives the prediction of lipophilicity and the core concept of the trespassing molecule across the BBB, higher log P values indicate more lipophilicity. Y3 indicated high value of LogP 6.953 in-comparison to piperine showing 3.313, and Y3 proved as lipophilic because of straight-chain structure, may mimicking Low-density lipoproteins thus it follows the LDL-transcytosis mechanism which might also a reason for BBB permeability [17]. A similar type of synthetic straight-chain imine conjugates was also reported to inhibit the MAO enzyme imparting antidepressant activity [18]. A high molar refractivity notes for a higher volume of interaction at the receptor site [19], as Y3 shown molar refractivity of 119.29 $cm^3$/mol in contrast to Piperine which has 85.91 $cm^3$/mol. Other parameter tPSA i.e. total polar surface area, which generally indicates the permeation ability of any drug into a cell, a higher value indicates less permeability [20]. Although tPSA of Y3 is shown to have a higher value of 47.56 Å in contrast to Piperine having 38.77 Å,

again LDL- transcytosis mechanism supports its efficient permeation into brain cells.

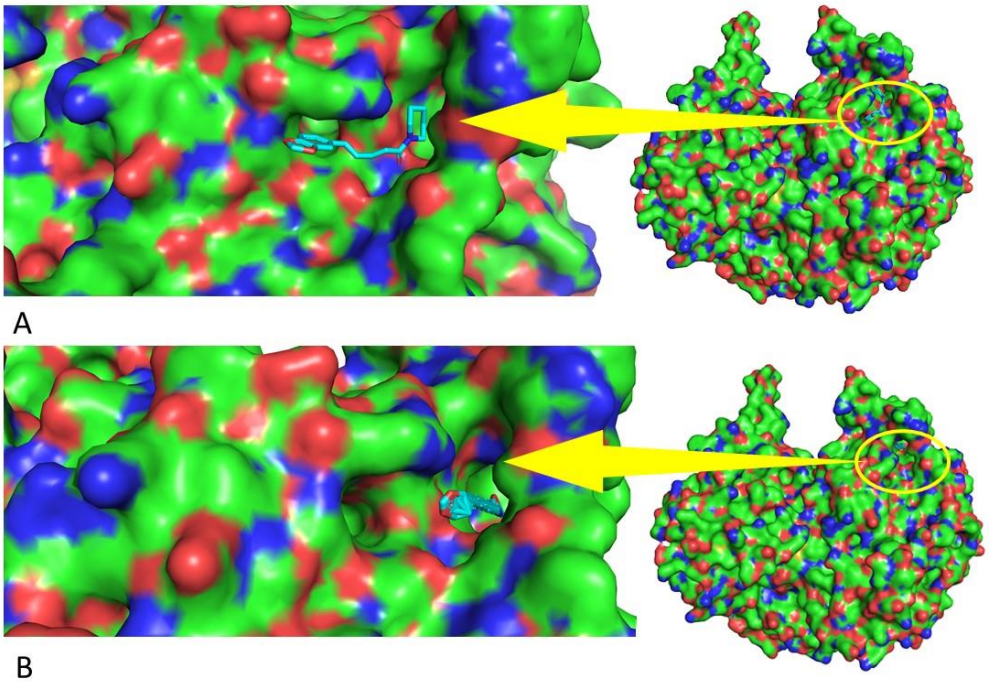


**Figure 3: A comparison between Piperine (A) and Y3 (B) at the MAO-A receptor site to depict molecule and receptor interaction. Both Piperine and Y3 sharing the same receptor pocket but different active sites.**

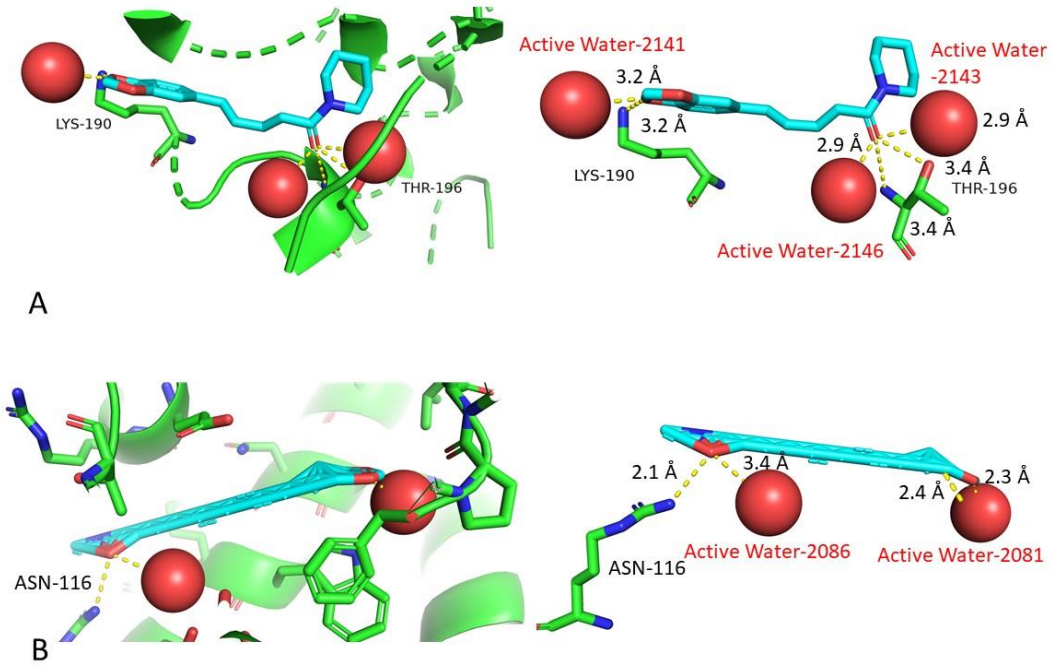


**Figure 4: A. Piperine's B. Y3's affinity towards MAO-A binding site.**

## CONCLUSION

A novel compound Y3 was isolated and elucidated as (12E,14E)-15-(benzo[d][1,3]dioxol-5-yl)-N-ethylpentadeca-12,14-dienamide. Y3 engraved better antidepressant activity via reducing the behavioral despair efficaciously than piperine at the dose 10

and 20 mg/kg. Molecular docking on MAO-A and QSAR parameters depicted a better affinity score of Y3 in contrast to Piperine. As behavioral despair is a common evaluation factor for Alzheimer's, Depression, Parkinson, and other neurological diseases. In the future, further work can be done to assess the therapeutic capability of Y3 in different neurological diseases.

## *Declaration by Authors*

**Ethical Approval:** Approved

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**Source of Funding:** None

**Conflict of Interest:** The authors declare no conflict of interest.

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